Part of #14/B

EXHIBIT "A"

Blockade of LIGHT/LTβ and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease

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Previous studies have shown that blockade of LIGHT, a T cell costimulatory molecule belonging to the TNF superfamily, by soluble lymphotoxin β receptor-Ig (LTβR-Ig) inhibits the cytotoxic T lymphocyte (CTL) response to host antigenic disparities and ameliorates lethal graft-versus-host disease (GVHD) in a B6 to BDF1 mouse model. Here, we demonstrate that infusion of an mAb against CD40 ligand (CD40L) further increases the efficacy of LTβR-Ig, leading to complete prevention of GVHD. We further demonstrate that alloantigen-specific CTLs become anergic upon rapid expansion, and persist in the tolerized mice as a result of costimulatory blockade. Transfer of anergic CTLs to secondary F1 mice fails to induce GVHD despite the fact that anergic CTLs can be stimulated to proliferate in vitro by antigens and cytokines. Our study provides a potential new approach for the prevention of lethal GVHD.

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Introduction

Graft-versus-host disease (GVHD) is caused by orchestrated alloreactive immune responses and is a major complication of bone marrow (BM) transplantation. Although ex vivo depletion of T cells can protect patients from severe GVHD, complications such as graft rejection, leukemia relapse, and delayed immune reconstitution can result from this approach (1). Pharmacological agents with immunosuppressive agents prevail as major therapeutic interventions in current clinical settings, but they require prolonged administration that can result in global immune suppression. It is highly desirable to induce a selective immunological unresponsiveness to host antigens that spares general T cell immunity against pathogens or residual leukemia cells.

Costimulatory receptor-ligand interactions play a critical role in the priming, growth, activation, differentiation, and death of T cells (2). Costimulatory blockade by either soluble receptor for or mAb against ligands has a profound effect and can lead to selective tolerance of T cells against alloantigens in some cases (3). Manipulations of B7-CD28 costimulatory interaction (4, 5) and of pathways belonging to TNF superfamily members such as CD40 and CD40 ligand (CD40L) (6), 4-1BB and 4-1BB ligand (7), OX40 and OX40 ligand (8), and LIGHT-HVEM (9) are capable of ameliorating GVHD to a certain degree and prolonging recipient survival. However, the mechanism accounting for T cell tolerance and the fate of host-reactive T cells upon costimulatory blockade are less understood.

Our previous studies demonstrated that LIGHT, a member of the TNF superfamily, provides potent costimulatory activity for T cells, enhancing proliferation and the production of Th1 cytokines independently of the B7-CD28 pathway (9, 10). Although LIGHT has three receptors – HVEM, lymphotoxin β receptor (LTβR), and DcR3/TR6 (11, 12) – HVEM is the primary receptor for T cell costimulation by LIGHT, since LTβR is not expressed on T cells (13) and DcR3/TR6 protein is found only in soluble form (14). Blockade of LIGHT-HVEM costimulation by either anti-HVEM mAb, HVEM-Ig, or LTβR-Ig fusion protein inhibits allogeneic T cell responses (9, 10, 15). Furthermore, in vivo administration of LTβR-Ig or anti-LIGHT Ab inhibits anti-host cytotoxic T lymphocyte (CTL) responses in a murine acute GVHD model, leading to improved survival of recipients (9).

Studies using anti-CD40L mAb and CD40L-deficient mice have demonstrated a critical role of CD40-CD40L interaction in the initiation, expansion, and maintenance of cell-mediated and humoral immune responses (16, 17). After stimulation with CD40, antigen-presenting cells, including B cells and dendritic cells, undergo maturation steps accompanied by increased

expression of MHC class II, B7-1, and B7-2, as well as secretion of IL-12 (18), leading to efficient triggering of T cell responses (19). Consistent with this notion, blockade of CD40-CD40L interaction has been shown to provide a therapeutic advantage for the prevention of acute and chronic GVHD (6), transplant rejection (20), and autoimmune diseases (21). In mouse models of acute GVHD, a brief treatment course with anti-CD40L mAb inhibits proliferation of and Th1 cytokine production by host-reactive $CD4^+$ T cells (22), and subsequently interferes with anti-host $CD8^+$ CTL generation (23). In addition, GVHD induced by CD28-deficient T cells has been shown to be inhibited by anti-CD40L mAb (24), suggesting that blockade of the CD40-CD40L pathway can decrease allogeneic responses independently of B7-CD28 costimulation. Ex vivo manipulation of donor T cells with anti-CD40L mAb efficiently generates host antigen-specific unresponsiveness and protects recipient mice from GVHD (25), although in vivo administration of anti-CD40L mAb was only partially effective in preventing GVHD lethality under the same GVHD conditions.

In this report, we describe a powerful method for achieving complete protection of recipient mice from acute GVHD and immunological tolerance using a combined administration of LTβR-Ig and anti-CD40L mAb. The mechanisms of tolerance induction by costimulatory blockade were explored.

Methods

Mice. Female C57BL/6J (B6, H-2^b), DBA/2J (H-2^d), and F1 (B6 × DBA/2J) (BDF1) mice were purchased from the National Cancer Institute (Frederick, Maryland, USA). Ly5-congenic B6.SJL-*$Ly5^a$ $ptprc^a$ $pep3^b$* (B6.Ly5.1), C.H-2^{bm1} (bm1), and C.H-2^{bm12} (bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). B6 2C (H-2^b) and B6 OT-I (H-2^b) T cell receptor (TCR) transgenic mice were developed by F. Carbone (Department of Microbiology and Immunology, Melbourne University, Victoria, Australia) and D.Y. Loh (Washington University, St. Louis, Missouri, USA), respectively. All mice were maintained in the Animal Facility at the Mayo Clinic.

Cell lines, fusion protein, and Ab's. P815 mouse mastocytoma cells (DBA/2, H-2^d), EL4 mouse T cell lymphoma cells (B6, H-2^b), and E.G7 cells (a chicken ovalbumin-transfected [OVA-transfected] EL4 line) (H-2^b) were purchased from the American Type Culture Collection (Rockville, Maryland, USA). AG104A sarcoma cells (H-2^k) were a gift from Hans Schreiber (University of Chicago, Chicago, Illinois, USA). All cell lines were maintained in a complete medium (9).

Mouse LTβR-Ig fusion protein was prepared as described (9). Anti-CD40L mAb was purified from the supernatants of hybridoma MR1 (26). Purified human IgG1 and hamster IgG were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and Rockland Immunochemicals Inc. (Gilbertsville, Pennsylvania, USA), respectively. Anti-2C TCR mAb was purified from the supernatants of hybridoma 1B2 (27) and further conjugated with phycoerythrin in our laboratory.

Mouse GVHD model. The mouse model of acute GVHD was described previously (9). Briefly, splenocytes (7×10^7) from B6 mice were injected intravenously into sublethally irradiated (6 Gy) BDF1 recipients on day 0. The irradiated recipient mice were intravenously administered 100 μg of anti-CD40L on day 0 or 100 μg of LTβR-Ig on days 0, 3, and 6, or a combination of both after transplantation. Hamster IgG and human IgG1 were used as controls for anti-CD40L and LTβR-Ig, respectively. The recipient mice were monitored for survival daily, and body weight was measured every 5 days. For CTL assay, the recipient spleen cells were harvested on day 7 and assessed by a standard ^{51}Cr release assay (28) without prior in vitro restimulation. Alternatively, B6.Ly5.1 spleen cells (7×10^7) were injected intravenously into irradiated BDF1 recipients, followed by treatment with anti-CD40L and LTβR-Ig as described. On day 9, B6 Ly5.1^+ donor cells were purified from recipient spleen cells using VarioMACS (Miltenyi Biotec, Auburn, California, USA), in which Ly5.1^+ cells were labeled with beads followed by trapping in LS column placed at magnetic fields. Purified Ly5.1^+ donor cells (2×10^6 cells/ml) were cultured with irradiated (30 Gy) DBA/2 spleen cells at 2×10^6 cells/ml for 5 days, and the CTL activity was assessed by a ^{51}Cr release assay.

For examination of $CD4^+$ T cell and $CD8^+$ T cell effects on isolated MHC disparities, bm12 or bm1 recipients were sublethally irradiated (6 Gy) and injected with purified $CD4^+$ (1×10^5 cells/recipient) or $CD8^+$ (3×10^5 or 1×10^6 cells/recipient) lymph node (LN) T cells from B6 mice. Either control Ig or LTβR-Ig (100 μg) was injected intraperitoneally or intravenously beginning on day -1 and continuing either every other day until day 21 or every 3 days until day 14 after transfer. No differences were noted in results from the two schedules of Ig treatment (data not shown), so data were pooled for analysis.

In the 2C T cell transfer model, 4×10^7 LN cells from 2C TCR transgenic mice were injected intravenously into sublethally irradiated BDF1 or B6 recipients on day 0; mice were subsequently treated with injections of anti-CD40L and LTβR-Ig as described above. On day 5 and day 15, recipient spleen cells were harvested and assessed for CTL activity against P815 cells without in vitro restimulation. The number of 2C T cells and their expression of CD62L were examined by flow cytometry.

In vivo transfer and in vitro culture of OT-I T cells. OT-I TCR transgenic LN cells, 20–40% of which are specific for an H-2^b-restricted OVA epitope (SIINFEKL), were combined with either B6.Ly5.1 or BDF1 spleen cells (3×10^7 cells each). These were then infused intravenously into irradiated BDF1 recipients. Mice were subsequently treated with anti-CD40L and LTβR-Ig as described above. Eight days after cell transfer, OT-I T cells in recipient spleens were enriched by depletion of Ly5.1^+ and H-$2K^{d+}$ cells by VarioMACS. The enriched

cells were plated at 1.5×10^6 cells/ml and stimulated with 10 ng/ml OVA peptide in the presence of irradiated B6 spleen cells at 1.5×10^6 cells/ml for 4 days. The CTL activity against non-pulsed EL4 cells, OVA peptide-pulsed (10 μg/ml) EL4 cells, and EG7 cells was assessed by a standard ^{51}Cr release assay.

Allogeneic BM reconstitution. To generate allogeneic BM-reconstituted mice, B6 BM cells were first depleted of T cells using the VarioMACS system with anti-Thy1.2 mAb-conjugated microbeads. Then lethally irradiated (11 Gy) BDF1 mice were injected intravenously with 5×10^6 T cell-depleted BM cells. Six weeks later, reconstitution of host lymphoid tissues by donor cells was confirmed by flow cytometry by double staining with H-2K^d and H-2K^b (data not shown).

Anti-host CTL induction in vitro and in vivo. Spleen cells from either naive B6 mice, long-term GVHD survivors, or B6 BM-reconstituted BDF1 mice were stimulated with irradiated (30 Gy) DBA/2 spleen cells (2×10^6 cells/ml each) in the presence or absence of 10 IU/ml of human IL-2 (Cetus Corp., Emeryville, California, USA). After 5 days, CTL activity against P815 cells was measured by a ^{51}Cr release assay. In addition, spleen cells (5×10^7 cells) from either naive B6 mice, long-term GVHD survivors, or B6 BM-reconstituted BDF1 mice were transferred intravenously on day 0 into secondary BDF1 recipient mice. In some groups, recombinant human IL-2 at a dose of 50,000 IU/day was injected intraperitoneally after spleen cell transfer. After 10 days, CTL activity of recipient spleen cells against P815 cells was assessed. Expansion of donor T cells and elimination of host B cells was quantified by flow cytometry by double staining with H-2K^d and CD3.

Statistical analysis. Group comparisons of continuous data were analyzed by Student t test. For survival data, Kaplan-Meier survival curves were prepared using StatView 5.0 software (SAS Institute Inc., Cary, North Carolina, USA), and statistical differences were analyzed using the log-rank (Mantel-Cox) test. P values lower than 0.05 were considered significant.

Results

Synergistic effects of LTβR-Ig and anti-CD40L mAb in the prevention of GVHD are accompanied by profound inhibition of anti-host CTLs. Upon transfer of B6 T cells, recipient BDF1 mice generate acute GVHD characterized by rapid weight loss, expansion of host-reactive donor T cells, shrinkage of the thymus, and eventual death (Figure 1, a and b, and data not shown). Infusion of LTβR-Ig, which blocks the LIGHT costimulatory pathway (9, 10), significantly reduced GVHD mortality as well as anti-host CTL activity. The treatment is not optimal because approximately 20% of mice still die of GVHD. However, combined treatment with anti-CD40L and LTβR-Ig in our study prevented death in 100% of recipient mice, whereas anti-CD40L treatment alone protected only about 50% of mice (Figure 1a). Combined therapy protected all recipient mice from death for more than 90 days (Figure 1a), and efficiently prevented weight loss in recipient mice (Figure 1b). This combined therapy also profoundly inhibited the generation of anti-host (H-2^d) CTL activity, whereas treatment by anti-CD40L alone did not affect CTL activity, and LTβR-Ig alone resulted in a partial inhibition (Figure 1c).

It has been suggested that the blockade of CD40-CD40L interaction prevents GVHD through a CD4$^+$ but not CD8$^+$ T cell-mediated mechanism (22). To investigate the differential effects of LTβR-Ig on CD4$^+$ and CD8$^+$ T cell-mediated GVHD, sublethally irradiated bm12 and bm1 recipients were injected intravenously with purified B6 CD4$^+$ and CD8$^+$ T cells, respectively. Infusion of LTβR-Ig significantly prolonged the survival of bm1 recipients of a minimum uniformly lethal dose of CD8$^+$ T cells (Figure 1d). In contrast, survival was not significantly prolonged in bm12 recipients given a minimum uniformly lethal dose of CD4$^+$ T cells (Figure 1e). Thus, LTβR-Ig treatment is more effective in inhibiting CD8$^+$ T cell-mediated GVHD than CD4$^+$ T cell-mediated GVHD under these conditions. Our results suggest that the synergistic effect of anti-CD40L mAb and LTβR-Ig in our model is mediated by inhibition of both CD4$^+$ and CD8$^+$ host-reactive T cells.

Combined therapy inhibits the generation of anti-host CTL activity without peripheral deletion of T cells. To trace T cells in the recipients after treatment with costimulatory blockade, we used 2C TCR transgenic T cells as donor cells. 2C T cells react specifically against H-2L^d antigen and express a defined T cell receptor that can be specifically identified (27). 2C T cells that were transferred into BDF1 mice expanded vigorously and generated a high level of anti-host CTL activity, as early as 5 days after cell transfer (Table 1). Similar to the results obtained from polyclonal T cell transfer shown in Figure 1c, injections of LTβR-Ig, but not anti-CD40L mAb, significantly inhibited CTL activity. Nearly complete inhibition of 2C CTL activity was observed in recipients treated with a combination of LTβR-Ig and anti-CD40L mAb. Inhibition of antigen-specific CTL generation was noted through day 15 after transfer, although cell counting taken on both day 5 and day 15 showed that 2C T cells in treated recipients had expanded as vigorously as those in control recipients (Table 1). Our data thus suggest that combined treatment with LTβR-Ig and anti-CD40L mAb inhibits anti-host CTL generation without deletion of host-reactive T cells.

After transfer into syngeneic B6 recipients, the percentage of 2C T cells expressing a low level of CD62L (CD62L^{low}), which indicates an antigen-experienced phenotype (29), remained constant in the range of 10–20% (Table 1). In contrast, the majority of 2C T cells transferred into BDF1 recipients converted to CD62L^{low} cells as early as 5 days after transfer. The combined therapy delayed downregulation of CD62L expression on 2C T cells, an effect that was accompanied by suppressed CTL activity. However, a significant number of 2C T cells had converted to CD62L^{low} cells by day 15, even though CTL activity remained low. A similar pattern was observed

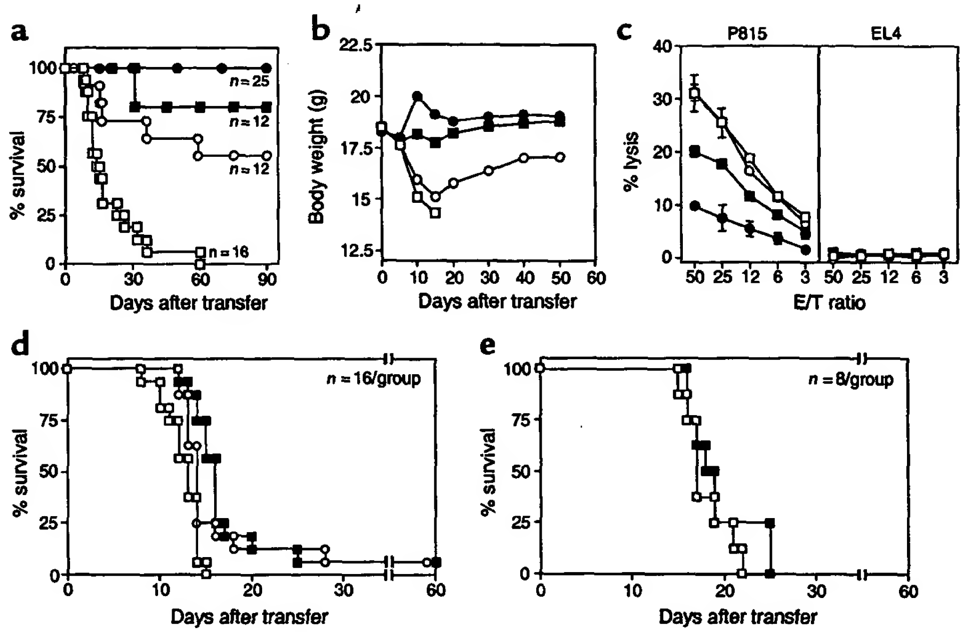

Figure 1
Synergistic effect of LTβR-Ig and anti-CD40L mAb in amelioration of GVHD and inhibition of anti-host CTL activity. (**a**–**c**) B6 splenocytes (7×10^7 cells) were injected into sublethally irradiated BDF1 mice and treated with either anti-CD40L (open circles), LTβR-Ig (filled squares), or both (filled circles). Hamster IgG and human IgG1 were injected as control (open squares). (**a**) Survival of recipients was examined daily, and pooled data from four independent experiments are presented. Treatment with both anti-CD40L and LTβR-Ig significantly prolonged survival compared to other treatments ($P < 0.05$). (**b**) Average body weight. Symbols same as for **a**. (**c**) The CTL activity of recipient spleen cells against P815 (H-2^d) and EL4 (H-2^b) was examined on day 7 without in vitro restimulation. Results are expressed as the mean ± SD of triplicate wells. (**d**) Purified B6 $CD8^+$ T cells (1×10^6 cells) were injected into sublethally irradiated bm1 mice followed by treatment with LTβR-Ig (filled squares) or control Ig (open squares) as described in Methods. Recipients of LTβR-Ig had a significantly ($P = 0.0002$) higher survival rate than did control-treated recipients. The reduction in GVHD lethality by LTβR-Ig treatment was estimated to be approximately equivalent to that resulting in control-treated mice from a threefold lower number (3×10^5) of $CD8^+$ cell transfer (open circles, $P > 0.1$). (**e**) Purified B6 $CD4^+$ T cells (1×10^5 cells) were injected into sublethally-irradiated bm12 mice followed by treatment with LTβR-Ig (filled squares) or control Ig (open squares) as described in Methods. No significant difference ($P > 0.1$) was noted between these two groups.

using CD44 marker (data not shown). Taken together, our results suggest that combined treatment with LTβR-Ig and anti-CD40L mAb inhibits the effector function, but is less effective at inhibiting the priming and expansion of host-reactive CTLs.

Combined therapy leads to repopulation of donor-derived lymphocytes in long-term GVHD-surviving mice. In the recipients that received the combined treatment and survived GVHD more than 60 days after transfer of B6 splenocytes, all lymphohematopoietic cells were H-$2K^{b+}$ and H-$2K^{d-}$, indicating complete replacement of the recipient lymphoid system by donor cells. Repopulation of donor lymphocytes was observed in spleen, LNs (Figure 2a), thymus (Figure 2b), and BM (data not shown). In addition, proportions of T cells, B cells, and $CD4^+$ and $CD8^+$ T cell subsets in spleen, LN, and thymocyte subsets were similar to those in naive B6 mice. Myeloid cells expressing Mac-1 also converted to donor-derived cells (data not shown). The proportion of $CD62L^{low}$ cells in recipient splenic and LN T cells, however, increased significantly compared with that in naive B6 mice (Figure 3a), suggesting constant exposure of T cells to host antigens. Similar results were also observed using 2C T cells as a donor source (Figure 3b). In this system, transferred 2C T cells were present more than 60 days in BDF1 recipients treated with the combined therapy and were comparable in number to those transferred into control B6 recipients. Importantly, a significant increase of $CD62L^{low}$ cells was detected in 2C T cells in BDF1 recipients compared with those in B6 recipients (Figure 3b). Our results thus demonstrate that donor-derived hematopoietic cells can repopulate in treated recipient mice in which antigen-experienced, host-reactive T cells persist long-term, without inducing GVHD.

Tolerance induced by combined therapy did not affect T cell responses to nominal antigens. To examine whether the tolerance is "infectious," we used OT-I T cells, which express a transgenic TCR that uniformly reacts with OVA antigen in the context of H-$2K^b$ (30), to facilitate isolation of antigen-reactive T cells. A mixture of OT-I T cells and B6.Ly5.1 splenocytes was transferred into BDF1 recipient mice, followed by combined treatment with LTβR-Ig and anti-CD40L mAb to induce tolerance. As a control, OT-I T cells mixed with BDF1 splenocytes were transferred into BDF1 recipients in which GVHD was not induced. Transferred OT-I T cells were recovered from recipient spleen by enrichment of a subpopulation that was negative for both H-$2K^d$ and

Table 1
Modification of 2C T cell functions by blockade of LIGHT and CD40L costimulators

Day after transfer	Recipient	Treatment	Percent lysis[A] 50:1	25:1	12.5:1	2C T cells[B] (×10^5)	Percent CD62L^low in 2C T cells
Day 5	B6	Control Ig	3.2 ± 0.6	1.2 ± 0.3	1.0 ± 1.1	8.6	18.6
	BDF1	Control Ig	25.2 ± 2.9	23.7 ± 0.5	20.0 ± 2.5	56.2	72.6
	BDF1	Anti-CD40L	30.9 ± 1.2	29.1 ± 1.2	22.7 ± 0.7	68.6	72.3
	BDF1	LTβR-Ig	15.4 ± 4.4	14.4 ± 4.1[C]	11.1 ± 3.0[C]	50.9	45.9
	BDF1	Anti-CD40L/LTβR-Ig	4.3 ± 2.1[D]	4.4 ± 1.3[D]	2.0 ± 1.1[D]	60.7	35.3
Day 15	B6	Control Ig	0.2 ± 0.5	0.4 ± 0.6	0.0 ± 1.3	18.8	15.1
	BDF1	Control Ig	12.6 ± 0.7	11.2 ± 1.8	8.6 ± 0.9	68.0	70.5
	BDF1	Anti-CD40L/LTβR-Ig	6.8 ± 0.8[D]	4.6 ± 0.5[D]	2.9 ± 1.3[D]	70.1	56.2

Sublethally irradiated B6 or BDF1 recipient mice were injected intravenously with 2C T cells and then treated with either anti-CD40L (100 μg) on day 0, LTβR-Ig (100 μg) on days 0, 3, and 6, or both treatments together. As a control, the same amount of hamster IgG and human IgG1 were injected into sublethally irradiated B6 or BDF1 mice on the same schedule. [A]CTL activity of recipient spleen cells against H-2^d target (P815) cells at the indicated effector:target ratio, measured without in vitro manipulation. [B]Number of 2C T cells was calculated by multiplication of spleen cell number by a percentage of 1B2$^+$ CD8$^+$ T cells (average of 2-3 recipients in each group). [C]$P < 0.05$, [D]$P < 0.01$ compared with BDF1 recipients treated with control Ig. Similar results were obtained in three repeated experiments.

Ly5.1. After in vitro restimulation with antigenic OVA peptide, OT-I T cells recovered from the tolerant recipients generated significant CTL activity against OVA peptide-pulsed EL4 cells and E.G7 cells, but not nonpulsed EL4 cells. CTL activity of these OT-I T cells was identical to that of OT-I T cells purified from control recipients (Figure 4). CTL activity of OT-I T cells can be inhibited when antigenic OVA peptide is administered to mice receiving transferred OT-I T cells (data not shown). These data demonstrate that T cell tolerance to allogeneic antigens induced by combined therapy does not inhibit the responsiveness of nonalloreactive T cells to other antigens.

Combined therapy induces an early and persistent anergy of host-reactive CTLs. The hallmark of T cell tolerance is its unresponsiveness to antigen in the presence of appropriate antigen-presenting cells (31). We first examined whether T cells isolated from tolerant mice can be induced to resume their CTL activity. As shown in Figure 5a, B6.Ly5.1 T cells, which were isolated from BDF1 mice 9 days after combined therapy and restimulated in vitro by allogeneic DBA/2 spleen cells as a source of H-2^d antigens, failed to lyse H-2^{d+} target cells. In contrast, B6.Ly5.1 donor cells transferred into syngeneic B6 recipients mounted considerable CTL activity in an H-2^d–specific manner after in vitro restimulation. Our results indicate that combined treatment with LTβR-Ig and anti-CD40L mAb can induce donor T cell anergy to allogeneic antigens in an early phase of T cell activation.

Host-reactive CTL activity was also assessed in tolerant mice more than 60 days after combined treatment was completed. No CTL activity against P815 target cells (H-2^d cells) was detected, using spleen cells from tolerant mice, when the cells were directly used as effectors without further restimulation (Figure 5b). Upon restimulation with irradiated splenocytes from DBA/2 mice as a source of antigen-presenting cells expressing H-2^d antigens, CTL activity specific to P815 target cells could be detected in spleen cells from tolerant mice. The level of CTL activity, however, was significantly lower than that in cells from the positive control, in which CTLs were generated from splenocytes of naive B6 mice 5 days after coculture with allogeneic DBA/2 spleen cells (Figure 5c). In addition, the decreased CTL activity in tolerant mice could be restored in vitro to a level comparable to that induced in naive B6 mice by

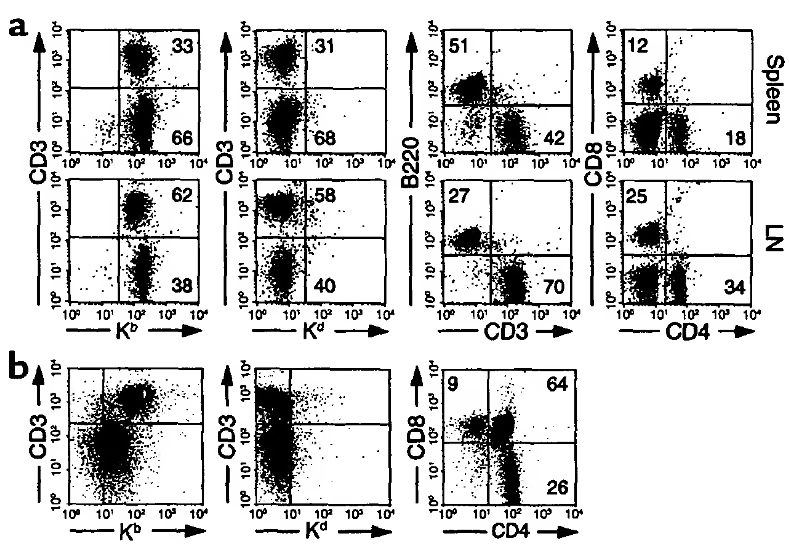

Figure 2
Repopulation of donor-derived lymphocytes in GVHD-surviving recipients. Sublethally irradiated BDF1 recipients were given B6 spleen cells (7 × 10^7 cells) and subsequently treated with a combination of anti-CD40L and LTβR-Ig. More than 60 days later, the recipient spleen cells, LN cells (**a**), and thymocytes (**b**) were stained with mAb's against indicated antigens conjugated with FITC or phycoerythrin and subsequently analyzed by flow cytometry. Similar data was obtained from eight independent mice surviving GVHD. Numbers in the figure represent the percentage of lymphocytes located in the same quadrants.

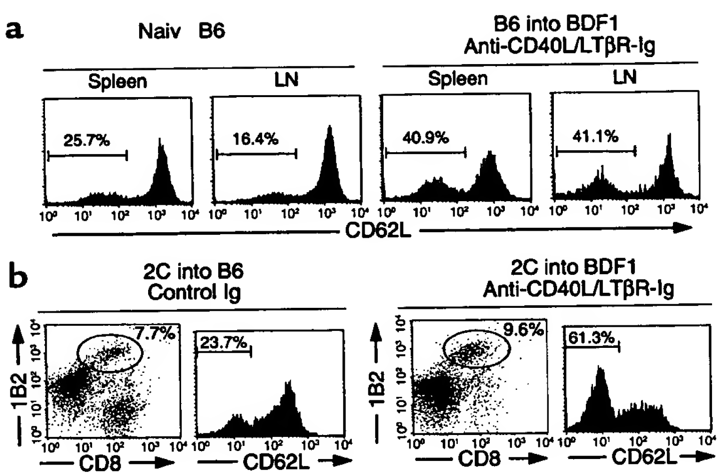

Figure 3
Persistence of host-reactive T cells in long-term GVHD survivors. (**a**) In naive B6 and GVHD-surviving mice (more than 60 days), spleen and LNs were stained with anti-CD3 and anti-CD62L mAb's and examined for CD62L expression of CD3-positive cells. (**b**) Sublethally irradiated B6 or BDF1 recipient mice received 4×10^7 LN cells from 2C TCR transgenic mice on day 0. BDF1 recipients were treated with anti-CD40L (100 μg, on day 0) and LTβR-Ig (100 μg on days 0, 3, and 6), whereas control Ig was injected into B6 recipients. More than 60 days later, recipient spleen cells were stained with anti-CD8, 1B2, and anti-CD62L mAb's. CD62L expression of $CD8^+1B2^+$ double-positive cells was examined. Numbers in the figure represent the percentage of 2C T cells (**b**) and the percentage of $CD62L^{low}$ cells (**a** and **b**).

addition of IL-2 (Figure 5d). As a negative control, spleen cells from BDF1 mice that had been reconstituted with T cell-depleted B6 BM cells did not induce any CTL activity (Figure 5c), probably because of negative selection of host-reactive T cells in the thymus.

Although cytolytic activity of host-reactive T cells in tolerant mice can be recovered in vitro by exposure to appropriate antigens, antigen-presenting cells, and cytokines, it is not known whether the same environment exists in vivo. To address this point, spleen cells from recipients tolerized by combined therapy were transferred into naive BDF1 recipient mice. Transfer of naive B6 splenocytes mediated profound $H\text{-}2^d$–specific CTL activity in the recipients. In contrast, BDF1 mice receiving splenocytes transferred from the tolerized mice or from B6 BM-reconstituted BDF1 mice did not generate CTLs (Figure 6a), in spite of a comparable number of T cells in transferred cells (Figure 2a). Administration of 50,000 IU/day IL-2 (Figure 6a) or 50 μg of LPS (data not shown) did not reverse the tolerant state of allogeneic T cells. Furthermore, vigorous expansion of donor T cells ($CD3^+H\text{-}2K^{d-}$), along with the elimination of host B cells ($CD3^-H\text{-}2K^{d+}$) — typical consequences of acute GVHD — were observed after transfer of naive B6 spleen cells but not after transfer of B6-derived cells present in BDF1 hosts tolerized by combined therapy or by B6 BM reconstitution (Figure 6b). Therefore, despite recovery of CTL activity by in vitro manipulation, anergic T cells in the mice given combined therapy remained tolerant in vivo even in the presence of corresponding antigens and exogenous IL-2.

Discussion

In this study, we have developed a new method to completely prevent acute GVHD in a B6 to BDF1 T cell transfer model. Combined administration of LTβR-Ig and anti-CD40L mAb induces complete, long-term (more than 90 days) survival of mice receiving immunocompetent allogeneic donor cells that without the combined treatment would induce acute lethal GVHD. In addition, we have demonstrated that the mechanism of this protection is the induction of persistent anergy rather than deletion of CTLs responding to alloantigens. Furthermore, donor-derived cells completely replace the recipient's hematopoietic system; this total replacement is associated with long-lasting unresponsiveness to host antigens. Anergy is specific for host-reactive CTLs but not for T cells reacting to other antigens. Our findings thus suggest a new approach to induce T cell anergy and to ameliorate GVHD in humans.

It is well-established in murine models that both $CD4^+$ and $CD8^+$ T cells are capable of mediating GVHD caused by disparities of MHC or minor antigens (32, 33). Taking into account the roles of $CD4^+$ T cells in the generation of alloreactive $CD8^+$ T cells (34), inhibition of the CD4 arm of the immune response may be neces-

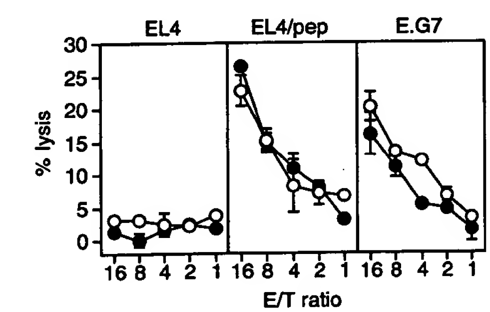

Figure 4
Intact T cell responses to nominal antigen in the combined therapy. Sublethally irradiated BDF1 mice received a mixture of OT-I LN cells (3×10^7 cells) and either 3×10^7 BDF1 spleen cells (open circles) or B6.Ly5.1 spleen cells (filled circles) on day 0. Recipients of transferred OT-I LN cells and B6.Ly5.1 cells received anti-CD40L (100 μg, on day 0) and LTβR-Ig (100 μg on days 0, 3, and 6). On day 8, cell populations negative for both Ly5.1 and $H\text{-}2K^d$ were enriched from recipient spleen cells by magnetic cell sorting. The purified cells (1.5×10^6 cells/ml) were stimulated with 10 ng/ml OVA peptide in the presence of irradiated B6 spleen cells (1.5×10^6 cells/ml) for 4 days. The CTL activity against nonpulsed EL4 cells, EL4 cells pulsed with 10 μg/ml of antigenic OVA peptide (EL4/pep), and E.G7 cells was assessed by ^{51}Cr release assay. Results are expressed as mean ± SD.

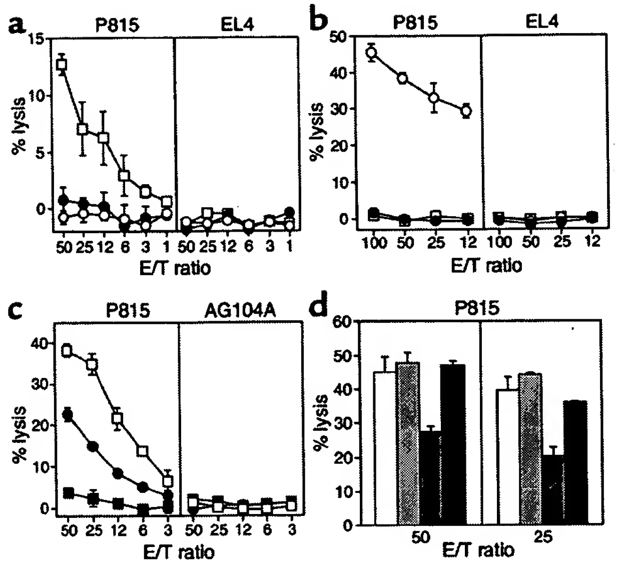

Figure 5
Induction of T cell anergy by combined treatment with LTβR-Ig and anti-CD40L mAb. (**a**) Sublethally irradiated BDF1 (filled circles) or B6 (open squares) mice were infused with B6.Ly5.1 splenocytes together with anti-CD40L and LTβR-Ig on day 0. On day 9, B6.Ly5.1$^+$ cells were purified and stimulated with irradiated DBA/2 splenocytes for 5 days. CTL activity against P815 and EL4 was assessed. The same recipient mice were injected with splenocytes from BDF1 mice as the controls (open circles). (**b**) CTL activity of splenocytes from recipients that survived GVHD more than 60 days was assessed against indicated targets without in vitro culture (filled circles). Splenocytes from BDF1 recipients that had received B6 (open circles) or BDF1 splenocytes (open squares) for 10 days were used as controls. (**c**) Splenocytes from recipients survived more than 60 days (filled circles) were stimulated for 5 days as described above, and subsequently examined for CTL activity against indicated targets. As controls, splenocytes from naive B6 mice (open squares) or B6 BM-reconstituted BDF1 mice (filled squares) were used as responder cells. (**d**) Splenocytes from recipients that survived (more than 60 days) were stimulated as described above in the absence (dark gray bars) or presence (black bars) of IL-2. Naive B6 splenocytes were similarly stimulated in the absence (white bars) or presence (light gray bars) of IL-2. After 5 days, CTL activity against P815 cells was examined. Results are expressed as the mean ± SD of triplicate wells. E/T ratio: ratio of effector cells to target cells in CTL assay.

sary to achieve the maximal effect. Several studies indicate that the primary role of anti-CD40L mAb in the reduction of GVHD lethality is to inhibit the functions of alloreactive CD4$^+$ T cells, such as expansion and Th1-type cytokine production (22), which could subsequently limit functional maturation of alloreactive CD8$^+$ T cells (23). In our study, a single dose of anti-CD40L mAb did not lead to inhibition of CTL activity in recipients receiving either transferred B6 spleen cells or 2C T cells (Figure 1c and Table 1), in spite of prolonged survival of approximately 50% of the recipient mice (Figure 1a). On the other hand, administration of LTβR-Ig significantly inhibited anti-host CTL activity, indicating a direct impairment of anti-host CD8$^+$ T cell function. Furthermore, administration of LTβR-Ig inhibited GVHD induced by CD8$^+$ T cells injected into bm1 mice, but not GVHD induced by CD4$^+$ T cells injected into bm12 mice. The synergistic effect, however, decreased to minimal in the GVHD model in which recipients received lethal radiation and subsequent transfer of T cell-depleted BM plus allogeneic spleen cells (data not shown). This may reflect decreased dependency on costimulation and CD4$^+$ T cells in lethal radiation-associated GVHD (35). Nevertheless, our results suggest a potential mechanism for the synergistic effects of LTβR-Ig and anti-CD40L mAb in the inhibition of GVHD.

Soluble receptor of LTβ is reported to bind at least two ligands belonging to the TNF superfamily, LIGHT and LTβ (11). Recent studies have demonstrated that signaling via LTβR by membrane LTβ is critical for the development and maintenance of secondary lymphoid structures (36) and for maintaining dendritic cells in secondary lymphoid tissues (37). Furthermore, cross-linking of LTβR induces the production of IL-8 and RANTES chemokine (38). Therefore, in addition to blockade of the LIGHT-HVEM costimulatory pathway, LTβR-Ig could have profound effects on both priming

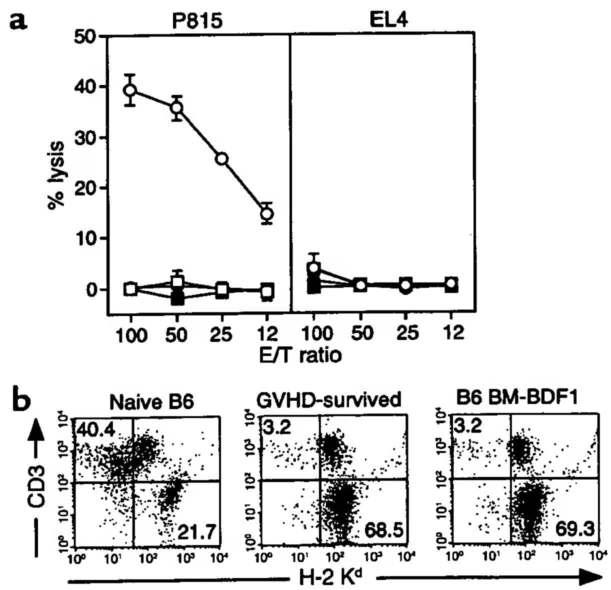

Figure 6
In vivo tolerance of host-reactive T cells in GVHD-surviving recipients. Spleen cells (5×10^7 cells) from recipient mice that survived GVHD for more than 60 days due to the combined treatment were injected intravenously into secondary BDF1 recipient mice on day 0 (open squares). In some mice, 50,000 IU of IL-2 was injected daily intraperitoneally from day 0 to day 10 (filled squares). As control, either naive B6 spleen cells (open circles) or spleen cells from B6 BM-reconstituted BDF1 mice (filled circles) were injected into BDF1 recipients. (**a**) After 10 days of cell transfer, recipient spleen cells were examined for CTL activity against P815 and EL4 cells without in vitro culture. Results are expressed as the mean ± SD of triplicate wells. (**b**) After 10 days of transfer of spleen cells from either naive B6 mice (left panel), GVHD-surviving mice (center panel), or B6 BM-reconstituted BDF1 mice (right panel), spleen cells of recipient mice were stained with mAb's against indicated antigens. Numbers in figure represent the percentage of lymphocytes located in that quadrant.

and migration of T cells. Our results indicating that administration of LTβR-Ig contributes to the induction of T cell tolerance suggests a new function of LTβR and HVEM receptor signaling in the regulation of peripheral T cell responses.

Induction of persistent T cell anergy is a unique treatment result, achieved by combined treatment with LTβR-Ig and anti-CD40L mAb. Anergic T cells from mice given combined therapy are not capable of responding to antigens after transfer into secondary recipients, even with IL-2 administration (Figure 6a). It has been reported that, upon transfer into recipient mice, 2C T cells undergo massive expansion by responding to H-2L^d-expressing host cells, followed by a rapid decline in cell number due to activation-induced cell death (39–41). The remaining apoptosis-resistant 2C T cells, although few in number, acquire an anergic phenotype, with abrogated responses to H-2L^d antigen and decreased expression of TCR and CD8 (39–41). Our present study demonstrates that combined therapy with LTβR-Ig and anti-CD40L mAb can inhibit the induction of CTL activity in 2C T cells, while still allowing the T cells to expand vigorously in the BDF1 host (Table 1). A similar process appears to occur in the B6 spleen cell transfer system, since donor cells repopulate the recipient mice (Figure 2).

T cells in the recipient mice exhibited an antigen-experienced phenotype with decreased CD62L expression and were unresponsive to antigen stimulation (Figure 3, Figure 5, and Figure 6), indicating that our combined therapy does not interfere with the induction of anergy. This is in remarkable contrast to the result of treatment with CTLA-4–Ig, since injection of CTLA-4–Ig during antigenic exposure often leads to T cells that are unactivated, but have a functionally competent status (immunological ignorance), presumably due to simultaneous blockade of CTLA-4 and CD28 (42). It has been shown that $CD4^+CD25^+$ regulatory cells contribute to the induction and maintenance of T cell tolerance (43), and that this mechanism is required for induction of tolerance to allogeneic antigens resulting from ex vivo blockade of CD40L or the B7-CD28/CTLA-4 pathway (44). Transfer of purified 2C $CD8^+$ T cells from tolerant mice into secondary recipients carrying H-2L^d antigen did not lead to activation of T cells (data not shown), suggesting that $CD4^+CD25^+$ T cells are not required for the maintenance of T cell anergy in this model. However, this observation does not exclude the possibility that $CD4^+CD25^+$ T cells may be involved in the induction of T cell anergy to allogeneic antigens in the early phase of the combined therapy.

The exact mechanisms by which our combined therapy induces persistent T cell anergy in vivo are currently unknown. Nevertheless, in our model, all hematopoietic cells are replaced by donor cells, whereas all somatic cells express host antigens, suggesting that nonhematopoietic cells in recipients may be involved in the maintenance of T cell anergy. It is possible that thymic epithelial cells in tolerant mice are still functionally competent to negatively select anti-host T cells from donors. Alternatively, constant exposure of T cells to peripheral somatic antigens might induce and maintain the anergy.

These mechanisms are not mutually exclusive. It is important that anergy of T cells appears to be specific, since the responsiveness of OVA TCR transgenic T cells remains normal when transferred into an environment lacking specific antigen (Figure 4). In addition, spleen cells from the tolerant mice did not inhibit anti-host CTL generation when cotransferred with naive B6 spleen cells into BDF1 mice (data not shown). Our results thus do not support mechanisms such as the production of suppressive cytokines by anergic T cells (45) or the generation of suppressive cells (41).

In vitro restimulation of spleen cells from the tolerant mice by allogeneic spleen cells induced low, but significant, CTL activity against host antigens (Figure 5c), a result consistent with restoration of CTL activity by inclusion of IL-2 in the cell culture (Figure 5d). Although IL-2 appears to reverse T cell anergy in various T cell culture systems, its effect in the regulation of T cell anergy in vivo is yet to be established. Dallman and colleagues showed that administration of recombinant IL-2 prevented development of tolerance of a renal allograft (46). However, several studies have demonstrated that the same approach was not effective in restoration of allogeneic CTL activity (25), a result similar to the results we present here (Figure 6a). Infusion of LPS, a potent inflammatory mediator, into the mice that were infused with anergic T cells also did not break tolerance in our model (data not shown), suggesting that a mechanism that operates in an in vitro system is not available in vivo. Taken together, our results indicate that combined treatment with LTβR-Ig and anti-CD40L mAb can prevent lethal GVHD by inducing T cell anergy in a mouse model and thus might represent a practical approach for translating costimulatory therapy of GVHD into clinical application.

Acknowledgments
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EXHIBIT "B"

Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway

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LIGHT was recently described as a member of the tumor necrosis factor (TNF) 'superfamily'. We have isolated a mouse homolog of human LIGHT and investigated its immunoregulatory functions *in vitro* and *in vivo*. LIGHT has potent, CD28-independent co-stimulatory activity leading to T-cell growth and secretion of gamma interferon and granulocyte–macrophage colony-stimulating factor. Gene transfer of LIGHT induced an antigen-specific cytolytic T-cell response and therapeutic immunity against established mouse P815 tumor. In contrast, blockade of LIGHT by administration of soluble receptor or antibody led to decreased cell-mediated immunity and ameliorated graft-versus-host disease. Our studies identify a previously unknown T-cell co-stimulatory pathway as a potential therapeutic target.

T lymphocytes often require two distinct signals to proliferate and to differentiate into effector cells that mediate immune responses[1]. In addition to antigen signals delivered by the T-cell receptor, the interaction between co-stimulatory receptors on T cells and their ligands on 'professional' antigen-presenting cells induce optimum activation and survival of T cells. Co-stimulatory pathways can be manipulated for therapeutic purposes. For example, blockade of the B7-CD28 co-stimulatory pathway by soluble cytotoxic T-lymphocyte antigen 4 immunoglobulin or monoclonal antibodies against B7 inhibits progress of autoimmune disease, the rejection of transplanted organs and graft-versus-host disease (GVHD)[2–4]. On the other hand, enhancement of co-stimulation by gene transfer of B7 or agonistic monoclonal antibody against the glycoprotein 4-1BB can increase anti-tumor immune responses[5,6].

LIGHT (name derived from: homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes; Genome Database designation, TNFSF14) was recently described as a member of the tumor necrosis factor (TNF) family[7], and its mRNA can be detected in most peripheral blood mononuclear cells after activation[7,8]. HVEM and lymphotoxin β receptor (LTβR) can bind LIGHT with high affinity[7]. Expression of HVEM can be detected in T, B and natural killer cells as well as endothelial cells[9,10]. In contrast, LTβR is not found in T and B cells, but is found in monocytes and stromal cells[11]. LIGHT triggers apoptosis of some tumor lines *in vitro* and *in vivo*, and the effect seems to require expression of both HVEM and LTβR on tumor cells[8], although these receptors lack typical death domain[12,13]. TR6 (DcR3) has also been suggested as a third receptor for LIGHT (ref. 14). A LIGHT fusion protein has been shown to stimulate a three-way mixed lymphocyte reaction (MLR) of peripheral blood mononuclear cells[15]; this effect is probably mediated through interaction with HVEM ligation. HVEM engagement signals through several TNF-receptor-associated factors, and overexpression of HVEM in 293 cells induces activation of nuclear factor-κB (refs. 16,17). In addition, blockade of HVEM by HVEM-immunoglobulin or a specific monoclonal antibody can inhibit allogeneic MLRs (refs. 9,10). These results indicate that interaction between LIGHT and HVEM is involved in the regulation of a T-cell response.

Here, we provide evidence that LIGHT is a CD28-independent co-stimulatory molecule for T-cell growth and differentiation. Moreover, we show that T-cell-mediated immune responses in tumor and in GVHD mouse models can be modulated through the LIGHT co-stimulatory pathway.

Molecular cloning of mouse LIGHT

We isolated a full-length cDNA of the mouse homolog of the human gene for LIGHT from Concanavalin A-activated mouse T cells by a combination of rapid amplification of cDNA ends and RT–PCR, and cloned it into a pcDNA3 mammalian cell expression vector (pmLIGHT). From the mouse cDNA for LIGHT, a protein of 239 amino acids can be deduced, with characteristics of a type II transmembrane protein and 77% amino acid homology with human LIGHT (Fig. 1*a*). The expected receptor-binding region of mouse LIGHT has substantial sequence homology with those of Fas ligand (33%), LTβ (30%), LTα (28%), TNF (27%), receptor activator of nuclear factor-κB ligand (26%) and TNF-related apoptosis-inducing ligand (23%) (data not shown). Transfection of pmLIGHT into 293 cells led to surface expression of LIGHT, as

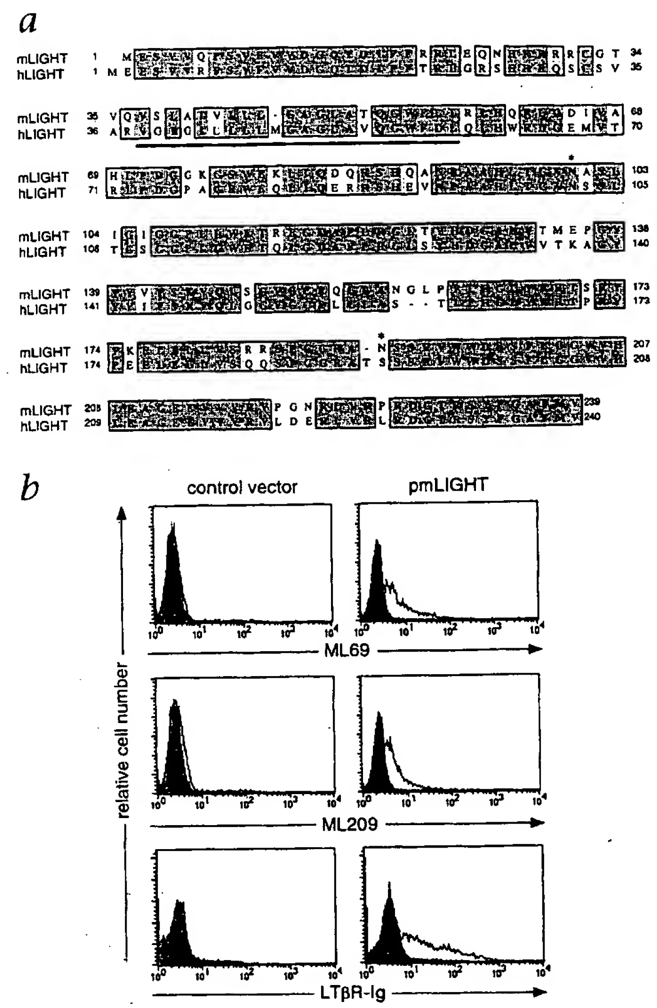

Fig. 1 Amino-acid sequence and expression of mouse LIGHT. ***a***, The amino-acid sequence of mouse LIGHT as deduced from the cDNA sequence, aligned with that of human LIGHT. To obtain the optimal alignment, several gaps (-) were introduced. Boxes, regions of homology; shading, identical residues; underlining, transmembrane region (TM); *, position of the predicted N-glycosylation site. Right and left ends, amino-acid numbers. ***b***, Human 293 cells transfected with the pmLIGHT or pcDNA3 were stained with antibody against LIGHT (ML69 or ML209) or LTβR–Ig, followed by FITC-conjugated goat antibody against rabbit IgG or human IgG, respectively (solid lines). Shaded areas, staining with rabbit IgG or human IgG1 (controls).

shown by staining with antibodies against LIGHT (ML69 and ML209) or a fusion protein of LTβR and immunoglobulin (LTβR–Ig) with flow cytometry analysis (Fig. 1*b*).

LIGHT co-stimulates CD28-independent T-cell responses

We determined whether LIGHT can function as a co-stimulatory molecule. For this, we used an *in vitro* co-stimulation assay with COS cells expressing LIGHT to stimulate purified mouse T cells in the presence of an immobilized monoclonal antibody against CD3 at a suboptimal dose. COS cells transfected with the pmLIGHT induced a substantial increase in T-cell proliferation compared with that of COS cells transfected with control vector (Fig. 2*a*). In the absence of antibody against CD3, COS cells transfected with pmLIGHT did not stimulate T-cell proliferation (Fig. 2*a*). This result indicates that LIGHT can co-stimulate T-cell growth in the presence of antigenic signal. Furthermore, immobilized fusion protein of LIGHT and the flag peptide sequence (LIGHT–flag) strongly stimulated a proliferation of purified mouse T cells in a dose-dependent manner in the presence of suboptimal amounts of antibody against CD3 (Fig. 2*b*). Like COS cells transfected with LIGHT (Fig. 2*a*), transfection with LIGHT–flag did not stimulate T cells in the absence of antibody against CD3 (Fig. 2*b*). In addition, the increased T-cell proliferation by LIGHT–flag was substantially blocked by antibody against LIGHT (ML209) and by LTβR–Ig (Fig. 2*c*). Our results show that LIGHT can co-stimulate T-cell growth when engagement of the T-cell receptor occurs.

We compared the ability of LIGHT–flag to co-stimulate proliferation of T cells isolated from CD28-deficient ($CD28^{-/-}$) mice and from normal littermates in the presence of monoclonal antibody against CD3. LIGHT–flag co-stimulated proliferation of $CD28^{-/-}$ and $CD28^{+/+}$ T cells to a similar degree, whereas the proliferative response to monoclonal antibody against CD28 in $CD28^{-/-}$ mice vanished (Fig. 2*d*). This result indicates that LIGHT can function independently of the CD28 co-stimulatory pathway.

Augmentation of T-cell-mediated tumor immunity by LIGHT

To test whether an increase in LIGHT co-stimulation can increase the cell-mediated immune response *in vivo*, we injected the pmLIGHT plasmid into established tumor nodules induced by P815 tumor cells and assessed the effect of LIGHT expression in the induction of cytotoxic T lymphocytes (CTLs) to tumor antigens and in the regression of the tumor nodules. When we inoculated mice subcutaneously with 2×10^5 P815 cells, palpable tumors developed, ranging in size from 3 to 5 mm (average diameter) in a week[18]. We repeatedly injected pmLIGHT (carried by liposomes) into tumor nodules, starting on day 7 after subcutaneous inoculation of tumor cells. We were able to detect expression of transfected LIGHT in tumor nodules by RT–PCR (data not shown). One week after the last plasmid injection, we stimulated spleen cells in a 5-day mixed lymphocyte–tumor culture and assayed them for CTL activity against P815 cells in a standard ^{51}Cr-release assay[5]. Mice treated with pmLIGHT had increased CTL activity against P815 cells compared with those treated with medium or the control vector (Fig. 3*a*, left). Although CTLs lysed P815 cells in large amounts, they did not kill L1210 cells in the same assay (Fig. 3*a*, right), indicating that the CTLs were specific for P815 tumor antigens. Our results show that LIGHT co-stimulation *in vivo* can increase the CTL response to P815 tumor antigen.

Repeated injections of pmLIGHT led to regression of tumors in all treated mice within 3 weeks from the beginning of injections (Fig. 3*b*), whereas the mice in the control groups treated with medium or control vector developed progressive growing tumors. Although injection of the control vector resulted in a slight slowing of tumor growth in some mice, probably because of nonspecific inflammation, all tumors in mice in the control groups grew progressively and eventually killed the mice. Depletion of $CD8^+$ T cells completely abrogated the anti-tumor effect of pmLIGHT, whereas the anti-tumor effect was partially inhibited by depletion of the $CD4^+$ T-cell subset (Fig. 3*c*). These results indicate that injections of pmLIGHT into P815 tumor nodules can eradicate tumors by a T-cell-dependent mechanism. Furthermore, P815 cells transfected to express LIGHT showed a substantial slowing of tumor growth when injected subcutaneously into mice (Fig. 3*d*). We obtained identical results using two independent LIGHT-positive p815 cell lines (data not shown). Our results support the idea that increased expression of LIGHT by tumor cells increases the immunogenicity of tumor.

The mice with regressed tumors after injections of pmLIGHT remained tumor-free for more than 45 days without recurrence (Fig. 3*b*). To determine whether they developed long-term tumor immunity, we challenged the mice with a lethal dose of P815 cells. These mice remained tumor-free after the challenge, whereas all naive mice developed tumors (Fig. 3*e*, left). The protection was specific for P815, as challenge of the mice with L1210 cells, an antigenically irrelevant syngeneic lymphoma, led to tumor growth at a similar speed in both groups (Fig. 3*e*, right).

Amelioration of acute GVHD by blockade of LIGHT

To determine the role of endogenous LIGHT in cell-mediated immune responses, we evaluated the effect of LTβR–Ig, a soluble receptor of LIGHT, in a mouse acute GVHD model. In this model, infusion of B6 (H-2b)-derived T cells into BDF1 (H-2bxd) mice, non-irradiated or irradiated with a dose sufficient to temporarily ablate the immune system, induces an acute GVHD accompanied by rapid weight loss, expansion of alloreactive donor T-cell populations, decreased numbers of host splenocytes, shrinkage of the thymus and rapid death of mice[19]. After receiving an intravenous injection of 7×10^7 splenocytes from B6 mice, mice received LTβR–Ig intravenously every 3 days beginning 1 day before splenocyte transfer (day –1). Treatment with LTβR–Ig improved the survival of recipient mice considerably (Fig. 4*a*), stopped their weight loss (Fig. 4*b*) and decreased alloreactive (against H-2d) CTL activity in spleens assayed at 11 days after B6 splenocyte transfer (Fig. 4*c*). In contrast, all mice treated with control immunoglobulin died within 2 weeks after splenocyte transfer, and had severe weight loss (Fig. 4*a* and *b*). Mice that underwent GVHD had more alloreactive CTL activity than control BDF1 mice that received splenocytes from BDF1 mice (Fig. 4*c*). Furthermore, the reduction in the numbers of host splenic B lymphocytes and double-positive thymocytes, which are typical consequences of GVHD, was considerably lessened by injections of LTβR–Ig (data not shown). Thus, our results show that LTβR–Ig can ameliorate alloreactive T-cell-mediated GVHD.

To assess the involvement of LTβ in our GVHD model, we also transferred splenocytes from LTα-deficient (LTα$^{-/-}$) B6 mice, in which both of LTα3 complexes and LTα1β2 heterotrimers (LTβ) are deficient[20], into non-irradiated BDF1 mice and measured the generation of anti-host CTL activity. BDF1 mice given splenocytes from LTα$^{-/-}$ mice showed an alloreactive CTL response similar to that generated by LTα$^{+/+}$ littermate control mice (Fig. 5*a*). The stimulation of naive LTα$^{-/-}$ B6 T cells by irradiated BALB/c splenocytes for 5 days induced a substantial alloreactive CTL activity against H-2d targets (Fig. 5*b*). Therefore, the *in vivo* as well as *in vitro* induction of alloreactive T cells is independent of lymphotoxins. Furthermore, the inclusion of LTβR–Ig, but not control immunoglobulin, in the culture substantially inhibited the alloreactive CTL response (Fig. 5*b*). These results show that LTβ production from donor cells is not required for alloreactive CTL responses in this GVHD model, and indicate that blockade of LIGHT by LTβR–Ig is responsible for the inhibition of alloreactive CTL-mediated GVHD. To test this, we injected BDF1 mice intraperitoneally with antibodies against LIGHT (ML69 and ML209) and assessed the alloreactive CTL activity 11 days after B6 splenocyte transfer. One of the antibodies, ML209, partially, but substantially, inhibited the generation of anti-host CTL activity (Fig. 5*c*). In contrast, neither a control antibody nor ML69 affected alloreactive CTL induction. Our results support the idea that LIGHT is involved in the induction of GVHD in our model.

Induction of IFN-γ and GM-CSF by LIGHT co-stimulation

We also studied the effect of LIGHT in differentiation of T cells. We stimulated purified T cells from B6 mice with immobilized LIGHT–flag in the presence of a suboptimal dose of antibody against CD3 for 2 days, and analyzed the culture supernatants for T-helper-cell type 1 and 2 cytokines by sandwich enzyme-linked immunosorbent assay (ELISA). LIGHT–flag co-stimulated the production of gamma interferon (IFN-γ) and granulocyte–macrophage colony-stimulating factor (GM-CSF) considerably, whereas secretion of interleukin (IL)-4 and IL-10 was not changed (Fig. 6*a*). We further assessed cytokine production in the culture supernatants of allogeneic MLRs. We used T cells purified from LTα-deficient splenocytes to exclude the possibility of an effect of lymphotoxin. The culture supernatants of allogeneic MLRs contained large amounts of IFN-γ and GM-CSF, whereas the amounts of IL-4 and IL-10 were either small or undetectable. The inclusion of LTβR–Ig substantially inhibited IFN-γ and GM-CSF production (Fig. 6*b*). Our results indicate that the LIGHT co-stimulatory pathway preferentially induces T-helper-cell type 1 responses, whereas blockade of LIGHT co-stimulation decreases T-helper-cell type 1 cytokine production.

Discussion

We have shown here that LIGHT is a co-stimulatory molecule for CD28-independent T-cell activation and preferentially induces IFN-γ and GM-CSF production. By using two mouse models, we

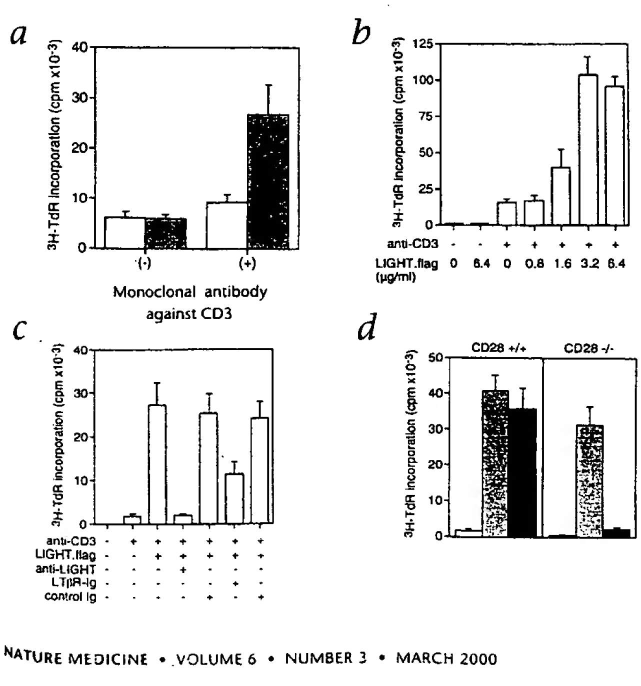

Fig. 2 Co-stimulatory activity of mouse LIGHT. ***a***, Purified T cells were stimulated with either irradiated COS cells transfected with pcDNA3 (□) or pmLIGHT plasmid (■) in the presence of antibody against CD3. ***b***, Purified T cells were stimulated with immobilized LIGHT–flag (doses, below graph) in the presence (+) or absence (-) of antibody against CD3 (anti-CD3). ***c***, Purified T cells were stimulated with 4 μg/ml immobilized LIGHT–flag and antibody against CD3 in the presence (+) or absence (-) of soluble antibody against LIGHT (ML209; anti-LIGHT) or LTβR–Ig. Equivalent doses of rabbit IgG and human IgG1 were used as controls for ML209 and LTβR–Ig, respectively. ***d***, Purified T cells (CD28$^{+/+}$ or CD28$^{-/-}$ splenocytes) were stimulated with 4 μg/ml immobilized LIGHT–flag (■) or soluble antibody against CD28 (■) in the presence of immobilized antibody against CD3. □, antibody against CD3 alone. Data represent ^{3}H-thymidine incorporation in counts per minute (c.p.m.) ± s.d.

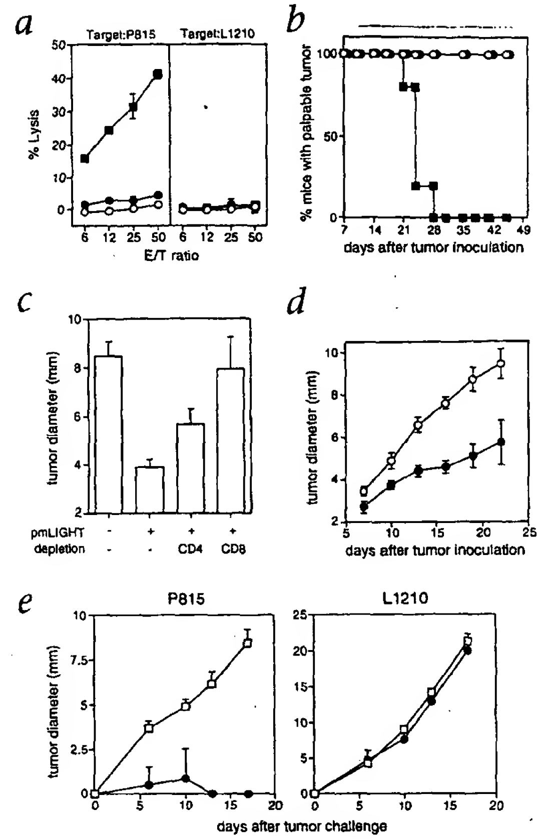

Fig. 3 Induction of immunity by LIGHT gene transfer against P815 tumor. ***a***, DBA/2 mice were inoculated with P815 cells at day 0 and then injected intratumorally with medium (○), pcDNA3 ( ) or pmLIGHT (■) on days 7, 9 and 11; 7 d after last injection, spleen cells were re-stimulated *in vitro* with irradiated P815 cells. The CTL activity was assessed by a standard ^{51}Cr-release assay against P815 and L1210 cells (effector:target (E/T) ratios, horizontal axis). Results are expressed as the means ± s.d. of triplicate wells; similar results were obtained in three independent experiments. ***b***, DBA/2 mice were inoculated subcutaneously with P815 cells on days 0: 7, 10, 14 and 17, mice with palpable tumor nodules were injected intratumorally with medium (○), pcDNA3 (●) or pmLIGHT (■). Results are expressed as percentage of mice with palpable tumor (more than 2 mm in average diameter). ***c***, DBA/2 mice were injected intraperitoneally with purified antibody against CD4 (GK1.5) or monoclonal antibody against CD8 (116-13.1) and inoculated subcutaneously with P815 cells on day 0. Medium (far left bar) or pmLIGHT (+) was injected intratumorally on days 7, 10, 14 and 17. Data represent tumor sizes on day 21 (mean diameters ± s.e.m.); similar results were obtained in two independent experiments. ***d***, DBA/2 mice were inoculated subcutaneously with mock-transfected (○) or LIGHT-transfected (●) P815 cells on day 0. Tumor sizes were measured every 3 d starting 7 d after tumor inoculation. Results are expressed as mean diameters ± s.e.m; similar results were obtained in two independent experiments. ***e***, Mice with regressed P815 tumors after pmLIGHT treatment were challenged with subcutaneous injection of P815 cells and L1210 cells 40 d after primary tumor inoculation (●). □, naive DBA/2 mice receiving both P815 and L1210 challenges (controls). Results are expressed as mean diameters ± s.d. of five mice in each group.

evaluated T-cell-mediated immune responses to P815 tumor and to allogeneic antigens in acute GVHD. LIGHT gene transfer stimulated tumor-specific CTL activity, leading to regression of established P815 tumors, and blockade of LIGHT by LTβR–Ig ameliorated GVHD. Our studies support the idea that LIGHT is involved in the induction of cell-mediated immune responses.

It remains to be determined whether LIGHT can serve as a co-stimulator in the priming or amplification of T-cell responses[21]. LIGHT can be detected on the surface of human T cells using soluble HVEM receptor[7]. A similar expression pattern of mouse LIGHT is also demonstrated by antibody against LIGHT; moreover, mouse dendritic cells also express LIGHT (K.T. *et al.*, unpublished data), indicating involvement of LIGHT in the interaction between dendritic and T cells. Although LIGHT can potentially bind three receptors[7,14] to interact with other cells, it is likely that HVEM is responsible for T-cell co-stimulation, as LTβR is not found on T cells[11] and the DcR3/TR6 protein does not have a transmembrane domain[14,22]. Although engagement of HVEM by LIGHT can potentially deliver a co-stimulatory signal, interaction between LIGHT and other receptors may be involved in immune regulation. LTβR is expressed by many non-lymphoid stromal cells of lymphoid tissues[11,23]. Cross-linking of LTβR by a specific monoclonal antibody or LTβ induces secretion of the chemokines IL-8 and RANTES (regulated upon activation, normal T-cell-expressed and -secreted)(ref. 24) and delivers a growth-inhibitory signal for some tumor lines *in vitro*[24,25]. Expression of DcR3/TR6 is increased in many lung and colon cancers, and DcR3/TR6 also binds to FasL (ref. 22). Thus, our results raise the possibility that some tumors can secrete soluble DcR3/TR6 to neutralize LIGHT as a mechanism of immune evasion.

LIGHT directly triggers apoptosis of some tumor cells in cell cultures, and transfection of LIGHT into a human breast carcinoma line led to its regression in T-cell-deficient athymic nude mice[8]. Only cells expressing both LTβR and HVEM are susceptible to the LIGHT-mediated apoptosis[8]. Nonetheless, it is unlikely that this apoptosis mechanism was functioning in our tumor system. P815 was negative for both LTβR and HVEM by RT–PCR analysis, and LIGHT-positive P815 cells are not apoptotic in culture (K.T. *et al.*, unpublished data). Moreover, the

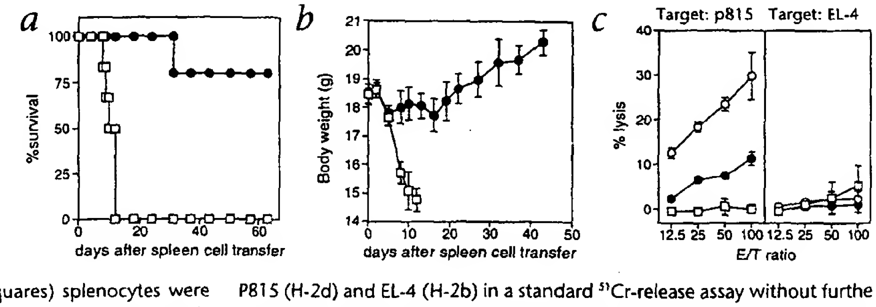

Fig. 4 Amelioration of GVHD by injection of LTβR–Ig. ***a*** and ***b***, BDF1 mice irradiated with a dose sufficient to temporarily ablate the immune system were injected intravenously with B6 splenocytes and given LTβR–Ig (●) or control human IgG1 (□) intravenously on days −1, 2, 5, 8, 11, 14 and 17 (control immunoglobulins were given until day 11 because all mice died within 12 days). Survival (*a*) and body weight (*b*; average ± s.e.m. of five mice in each group) were monitored daily. ***c***, Non-irradiated BDF1 mice injected intravenously with B6 (circles) or BDF1 (squares) splenocytes were given LTβR–Ig (●) or control human IgG1 (○) intravenously on days −1, 1, 3, 5, 7, 9. On day 11, splenocytes were assayed for their CTL activity against P815 (H-2d) and EL-4 (H-2b) in a standard ^{51}Cr-release assay without furthe stimulation *in vitro*. Horizontal axes, effector:target (E/T) ratios. Results are expressed as the means ± s.d. of triplicate wells.

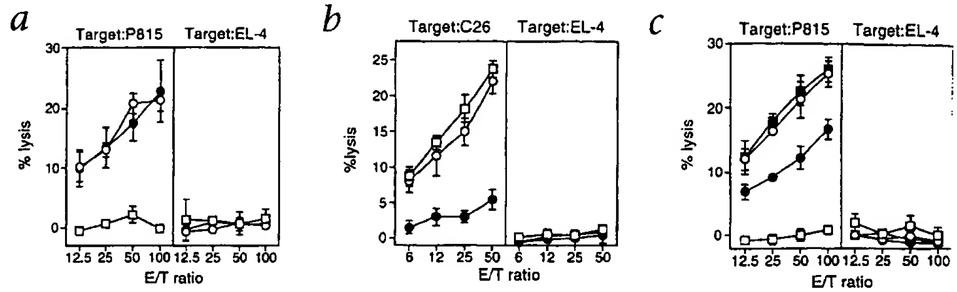

Fig. 5 Inhibition of alloreactive CTL induction by blockade of LIGHT pathway. ***a***, Non-irradiated BDF1 mice were injected intravenously with B6 LTα$^{-/-}$ ( ) or LTα$^{+/+}$ (○) splenocytes on day 0. □, BDF1 mice injected with BDF1 splenocytes (controls). On day 11, CTL activity of recipient splenocytes was assessed against P815 (H-2d) and EL-4 (H-2b) in a standard ^{51}Cr-release assay without further stimulation *in vitro*. ***b***, Purified T cells of B6 LTα$^{-/-}$ splenocytes were cultured with irradiated BALB/c splenocytes in the presence of LTβR–Ig (●), control human IgG1 (○) or no immunoglobulin (□). After 5 d, the CTL activity against C26 (H-2d) and EL-4 (H-2b) was measured in a standard ^{51}Cr-release assay. ***c***, Non-irradiated BDF1 mice injected intravenously with B6 splenocytes on day 0 were given antibody against LIGHT (ML69, ■ or ML209, ●) or control rabbit IgG (○)on days –1, 2, 5, 8. □, BPF, mice injected with BDF1 splenocytes (controls). On day 11, CTL activity of recipient splenocytes was assessed against P815 (H-2d) and EL-4 (H-2b) in a standard ^{51}Cr-release assay without further stimulation *in vitro*. Results are expressed as the mean ± s.d. of triplicate wells and represent one of three experiments. Horizontal axes, effector:target (E/T) ratios.

tumor-inhibitory effect of LIGHT DNA injection could be completely abrogated by the depletion of CD8-expressing T cells (Fig. 3*c*). Our results indicate that immune responses are mainly responsible for the anti-tumor effect. Transfection of LIGHT plasmid into P815 tumor cells decreased their tumorigenicity (Fig. 3*d*), indicating an enhancement of the co-stimulatory activity of tumor cells, although the possibility that LIGHT DNA is incorporated into and expressed on antigen-presenting cells to indirectly provide co-stimulatory activity after DNA injection cannot be excluded.

Monoclonal antibody against HVEM can significantly inhibit the proliferation of T cells in allogeneic MLR *in vitro*[9], indicating that endogenous interaction between LIGHT and HVEM is required for the induction of allogeneic T cells. Our study extends this observation and shows that infusion of LTβR–Ig prevented the onset of GVHD in F1 recipient mice and inhibited allogeneic CTL activity (Fig. 4). Although these results can be interpreted as being the consequence of a blockade of endogenous LIGHT by LTβR–Ig, there are several alternative interpretations. Infusion of LTβR–Ig inhibits the lymph node genesis, prevents the development of normal spleen architecture[26] and decreases the number of follicular dendritic cells, leading to impaired humoral immune responses[27]. GVHD, however, can be induced in TNFRp55$^{-/-}$ mice, in which disrupted follicular dendritic cell networks and impaired spleen architecture are evident[28]. Transfer of LTα$^{-/-}$ donor cells, in which both LTα and membrane-bound LTβ are deficient[20], still generated an anti-host CTL response *in vivo* (Fig. 5*a*), indicating that a complex of LTα or LTαβ is not required for the induction phase of GVHD. Moreover, an alloreactive CTL response can be normally generated from LTα-deficient mice *in vitro* (ref. 29 and Fig. 5*b*), again indicating that lymphotoxins are not required in this system. Furthermore, the inclusion of LTβR–Ig completely abrogated the generation of allogeneic CTLs from LTα-deficient T cells in the MLR assay. Therefore, it is unlikely that LTβ is involved in the induction of GVHD shown here, although it is still possible that LTβR–Ig could interact with an unknown ligand other than LTβ or LIGHT. Nonetheless, injections of antibody against LIGHT partially, but substantially, inhibited the induction of allogeneic CTLs (Fig. 5*c*), supporting the idea of the involvement of LIGHT in the induction of GVHD. The effectiveness of ML209 but not ML69 in blocking the induction of GVHD may be due to its ability to occupy a receptor binding site. The consensus loop structures comprising β strands of TNF-β, CD40 ligand and TNF-related apoptosis-inducing ligand make contact with the receptor[30–32]. The loop structure starts from amino acid 90 of LIGHT protein. Therefore, it is unlikely that ML69 antibody competes with HVEM receptor.

In conclusion, our study indicates that LIGHT is involved in cell-mediated immune response, and that manipulation of the LIGHT co-stimulatory pathway might be used in the treatment of diseases such as cancer, transplantation and autoimmune diseases.

Methods

Mice. Female C57BL/6 (B6), BALB/c, DBA/2 and F1(B6 × DBA/2) (BDF1) mice were purchased from the National Cancer Institute (Frederick, Maryland). CD28$^{-/-}$ mice (provided by M. Rodriguez) and LTα$^{-/-}$ mice with a B6 background and their corresponding littermates have been described[29,33].

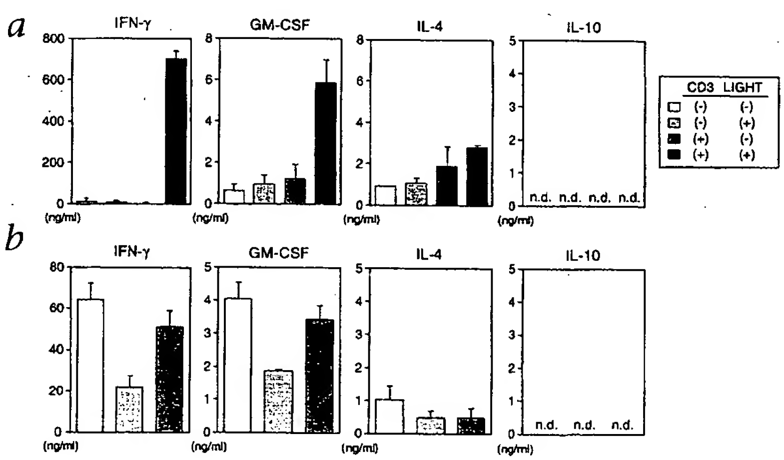

Fig. 6 Cytokine production by modulation of LIGHT co-stimulatory pathway. ***a***, Purified T cells were stimulated with immobilized LIGHT–flag in the presence (+) or absence (-) of immobilized antibody against CD3. After 48 h, cytokine production of culture supernatants was assessed by sandwich ELISA. ***b***, T cells purified from B6 LTa$^{-/-}$ splenocytes were cultured with irradiated BALB/c splenocytes in the presence of LTβR–Ig (■), control human IgG1 (▩) or no immunoglobulin (□). After 72 h, cytokine production in culture supernatants was assessed by sandwich ELISA. n.d., IL-10 was not detectable (less than 0.3 ng/ml) in any wells.

Cell lines. COS, 293 human kidney epithelial cells, P815 mouse mastocytoma, L1210 mouse lymphoma and EL-4 mouse T-cell lymphoma were purchased from the American Type Culture Collection (Rockville, Maryland). The C26 mouse colon cancer line was provided by R. Evans (The Jackson Laboratory, Bar Harbor, Maine). Cell lines were maintained in a complete medium of RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), 25 mM HEPES, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate.

Isolation of mouse LIGHT cDNA. Mouse LIGHT cDNA was isolated by PCR using degenerate primers of human LIGHT. The sense primer (5′–TTTCGTI-GATCGGICA–3′; I, inosine) has a sequence corresponding to the human LIGHT sequence at 31-47. The antisense primer (5′–AGGATGIACIACIC-CICC–3′) corresponds to the human LIGHT sequence at nucleotides 609–626. Poly(A)+ RNA from the spleens of BALB/c mice was reverse-transcribed and subjected to PCR with the primers described above. The sequence of the PCR product was highly homologous to that of human LIGHT cDNA, and was used to isolate the missing 5′ end and 3′ end by rapid amplification of cDNA ends using the Marathon™ cDNA Amplification kit (Clontech, Palo Alto, California). The full-length cDNA of mouse LIGHT was cloned into pcDNA3 vector (Invitrogen, Carlsbad, California). The homology between mouse and human LIGHT was analyzed using the ClustalW program of Macvector 6.5.

Fusion proteins and antibodies. To prepare the mouse LTβR–Ig fusion protein, cDNA encoding mouse LTβR extracellular domain was generated by RT–PCR using the sense primer 5′–AAAGGCC GCCATGGGCCT–3′ and the antisense primer 5′–TTAAGCTTCAGTAGCATTGCTCCTGGCT–3′ from mouse lung mRNA. After being digested with *NcoI*/*HindIII*, the PCR product was fused with an IL-3 leader sequence in the p30242 vector and then subcloned into a pX58 vector containing an IE-175 promoter and the Fc portion of human IgG1. The construct was then transfected into BHK/VP16 cells and mouse LTβR–Ig was purified from the conditioned media by a Sepharose–protein A affinity column. Fractions were then separated by SDS–PAGE through 4–20% gradient gels to confirm the presence of the desired protein and were further dialyzed against phosphate-buffered saline. After purification, the resultant mouse LTβR–Ig was more than 95% pure, as determined by Coomassie blue staining of an SDS–PAGE gel.

To produce the LIGHT–flag fusion protein, a cDNA encoding mouse LIGHT extracellular domain was fused with the flag peptide sequence, and was expressed in inclusion bodies in *Escherichia coli*. After being solubilized in 6 M guanidine hydrochloride, the proteins were subsequently diluted and allowed to fold. The folded protein was then purified to near-homogeneity by cation exchange chromatography. The final product was more than 95% pure, as determined by Coomassie blue staining of an SDS–PAGE gel. The endotoxin concentration was less than 1 pg/mg of purified protein according to limulus amebocyt lysate assays (CAPE COD, Woods Hole, Massachusetts).

The rabbit antibodies against mouse LIGHT peptide (ML69 and ML209) were prepared by the Cocalico Biologicals (Reamstown, Pennsylvania) by immunization with keyhole limpet hemocyanin-conjugated synthetic peptides encoding mouse LIGHT amino acids 69–91 (HLPDG-GKGSWEKLIQDQRSHQANC) and 209–232 (CEAGEEVVVRVPGNRLVR-PRDGTRS), respectively. The antibodies were purified from serum using peptide-conjugated affinity columns. ELISA and fluorescence-activated cell sorting analysis demonstrated that these antibodies bind mouse LIGHT but not FasL, LTβ, LTα or TNF-α (data not shown). Monoclonal antibodies against CD4 (GK1.5) and CD8 (116-13.1) were purified from hybridoma supernatants as reported[6]. Purified monoclonal antibodies against mouse CD3, CD28 and fluorescein isothiocyanate (FITC)-conjugated CD4 and CD8 were purchased from PharMingen (San Diego, California). FITC-conjugated goat antibodies against human and rabbit were purchased from Biosource International (Camarillo, California) and Southern Biotechnology Associates (Birmingham, Alabama), respectively. Purified human IgG1 and rabbit IgG were purchased from Sigma and Rockland (Gilbertsville, Pennsylvania), respectively.

Flow cytometric analysis. Human 293 cells (1×10^6 cells) were transfected with 10 μg mouse LIGHT pcDNA3 vector or 10 μg 'mock' (control pcDNA3 vector or parental pcDNA3 vector) using the calcium phosphate method. After 30 h of transfection, 293 cells were collected and stained for 30 min at 4 °C with 1 μg antibody against LIGHT or mouse LTβR–Ig in 50 μl phosphate-buffered saline supplemented with 3% fetal bovine serum and 0.02% azide. The cells were then washed and further incubated for 30 min at 4 °C with FITC-conjugated antibody against rabbit IgG or human IgG, respectively. Fluorescence was analyzed by a FACSCalibur flow cytometry with Cell Quest software (both from Becton Dickinson, Mountain View, California).

T-cell co-stimulation assay. T cells were positively selected by FITC-conjugated monoclonal antibodies against CD4 and CD8 using anti-FITC microbeads in the magnetic field as instructed by the manufacture (Miltenyi Biotec, Auburn, California). The purity of isolated T cells was greater than 95%, as assessed by flow cytometry using monoclonal antibody against CD3. Purified T cells (1×10^6 cells/ml) from mouse spleens were stimulated with plate-coated monoclonal antibody against CD3 (2 μg/ml) in the presence of COS cells (5×10^4 cell/ml) irradiated with 150 Gy, which had been transfected for 48 h with pcDNA3 or pmLIGHT using the DEAE–dextran method. Alternatively, plates coated with 0.2 μg/ml monoclonal antibody against CD3 were further coated at 37°C for 4 h with LIGHT–flag. After being washed, T cells (1×10^6 cells/ml) purified from spleens of B6 mice, $CD28^{-/-}$ mice or their littermates were cultured in the wells in the presence or absence of soluble antibody against LIGHT (ML209; 10 μg/ml) or LTβR–Ig (20 μg/ml). Monoclonal antibody against CD28 (1 μg/ml) was used in soluble form. In all assays, the proliferation of T cells was assessed by the addition of 1 μCi/well ^{3}H-thymidine during the last 15 h of the 3-day culture. ^{3}H-thymidine incorporation was measured in a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

Gene transfer into P815 cells and CTL assay. DBA/2 mice (n = 5–10 per group) were inoculated subcutaneously with 2×10^5 P815 cells. One week later, the mice with palpable tumors 3- to 5-mm in average diameter were treated. To prepare LIGHT DNA for *in vivo* injection, 10 μg pmLIGHT and 25 μg cationic liposome (DMRIE-C; Life Technologies), were mixed in 50 μl Opti-MEM I (Life Technologies) and incubated in polystyrene tubes at room temperature for 30 min. The complex was injected intratumorally on days 7, 10, 14 and 17 after tumor inoculation. Control mice were injected as described above with 50 μl Opti-MEM I or a mixture of 10 μg parental pcDNA3 and 25 μg DMRIE-C in 50 μl Opti-MEM I. For *in vivo* depletion of lymphocytes, mice were injected intraperitoneally with 500 μg/mouse purified monoclonal antibody against CD4 or CD8 1 d before tumor inoculation and every 2 weeks as reported[5,6,18]. Tumor sizes were determined by measuring the perpendicular diameters every 3 or 4 d.

DBA/2 mice, which had regressed P815 tumors after pmLIGHT injections, were re-challenged subcutaneously with 2×10^5 P815 cells in the right side of the back and the same number of L1210 cells in the left side of the back 40 d after primary tumor inoculation. Naive DBA/2 mice receiving with 2×10^5 P815 and L1210 cells were used as controls. Afterwards, tumor sizes were determined by measuring perpendicular diameters[5].

For the CTL assay, DBA/2 mice were inoculated with 2×10^5 P815 cells subcutaneously and then treated on days 7, 9 and 11 with pmLIGHT or control vector (parental pcDNA3 vector). Spleens were collected on day 18. Splenocytes (5×10^6 cells/ml) were cultured for 4 d in the presence of P815 cells irradiated with 150 Gy, and then assayed for CTL activity in a standard ^{51}Cr release assay[5]. After 4 h of incubation, 100-μl aliquots of supernatants were collected and their radioactivity was measured in a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

To establish LIGHT-transfected tumors, P815 cells were transfected with the pmLIGHT plasmid by electroporation. The transfected cells were selected in complete medium containing 1 mg/ml G418 (Life Technologies). G418-resistant P815 cells were cloned by limiting dilution. LIGHT-positive cells identified by their expression of LIGHT RNA and protein were used for experiments. DBA/2 mice were inoculated subcutaneously with 2×10^4 LIGHT-transfected P815 cells, and the tumor sizes were measured every 3 d starting at 7 d after tumor inoculation. P815 tumor cells transfected with pcDNA3 vector were used as controls.

The GVHD model and allogeneic CTL assay. For the induction of acute GVHD, 7×10^7 splenocytes from B6 mice were injected intravenously on day 0 into BDF1 mice that were non-irradiated or irradiated with a dose (4

Gy) sufficient to temporarily ablate the immune system. The irradiated recipient mice were given 100 μg LTβR–Ig or human IgG1 (control) intravenously 1 d before splenocyte injection (day −1) and every 3 d for six additional doses. Mouse survival and body weight were monitored daily. For the CTL assay, non-irradiated recipient mice were given 100 μg LTβR–Ig or control immunoglobulin intravenously on days −1, 1, 3, 5, 7 and 9. Alternatively, 500 μg antibody against LIGHT (ML69 or ML209) or control rabbit IgG was injected intraperitoneally into recipient mice on days −1, 2, 5 and 8. On day 11, the CTL activity of the recipient splenocytes was determined by co-culture with labeled P815 (H-2d) or EL-4 (H-2b) in a standard ^{51}Cr-release assay. Splenocytes from BDF1 mice that received BDF1 splenocyte transfer were used as a negative control. Identical experiments were also done using splenocytes from B6 $LT\alpha^{-/-}$ mice; age-matched B6 mice were used as controls in this experiment.

In vitro induction of CTLs to alloantigens in MLR was done as described[29]. Magnetic bead-purified T cells of B6 $LT\alpha^{-/-}$ mice were co-cultured at a concentration of 1×10^6 cells/ml, in the presence or absence of 25 μg/ml LTβR–Ig or control human IgG1, with the same number of BALB/c splenocytes irradiated with 30 Gy. After 5 d, the CTL activity against C26 (H-2d) and EL-4 (H-2b) was determined in a standard ^{51}Cr-release assay.

Cytokine assay. Purified B6 T cells (1×10^6 cells/ml) were stimulated with 3.2 μg/ml plate-coated LIGHT–flag in the presence of 0.5 μg/ml antibody against CD3 as described above. The culture supernatants were collected at 48 h and assayed by sandwich ELISA for IFN-γ, GM-CSF, IL-4 and IL-10 following the manufacture's instructions (PharMingen, San Diego, California). For assaying cytokine production in allogeneic MLRs, purified B6 $LT\alpha^{-/-}$ T cells at a concentration of 1×10^6 cells/ml were stimulated with the same number of irradiated BALB/c splenocytes in the presence or absence of 25 μg/ml LTβR–Ig and control human IgG. The culture supernatants were collected at 72 h and assayed for IFN-γ, GM-CSF, IL-4 and IL-10 production by ELISA.

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